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(57) Abstract

Oligonucleotides having tandem sequences of inverted polarity, i.e., oligonucleotides comprising regions of the formula: 3'-----5'--C--5'-----3' or 5'-----3'--C--3'-----5', wherein -C- symbolizes any method of coupling the nucleotide sequence of opposite polarity, are useful for forming an extended triple helix with a double-helical nucleotide duplex. Single or mixed motif oligomers may be used. The inverted polarity also stabilizes the single-strand oligonucleotides to exonuclease degradation.

TRIPLE HELIX FORMATION IN OLIGONUCLEOTIDE THERAPY

Technical Field

The invention is directed to modes of therapy which utilize oligomers designed to form triple helices with duplex DNA. More specifically, the invention concerns provision of pharmaceutical compositions containing oligonucleotides which target the major grove of the DNA duplex.

The invention is also directed to oligonucleotides having tandem sequences of inverted polarity, which are useful for forming triple helices with double-stranded duplex DNA. The inverted polarity oligonucleotides may be stabilized by this inversion which presents an unnatural terminus or internal linkage, thereby avoiding potential damage by nucleases.

Background Art

The rules which govern the association of single-stranded oligonucleotides with DNA duplexes to form triple-helical complexes have been recently described. At present, there are two recognized motifs for effecting triple helix formation. The older of these, commonly referred to as the "CT" motif, provides for a single-stranded oligomer containing, in its essential recognition portions, pyrimidine-based sequences which will result in T-A-T and C-G-C^+ based triplets across the three associated chains of the resulting triple helix. This system is effective when there are long stretches in the duplex wherein one of the two strands contains only, or mostly, purine base

instances, a concentration of purine residues along a portion of a single strand of the targeted duplex is required. Nevertheless, it is advantageous to have available a repertoire of strategies for targeting duplexes which permits advantage to be taken of additional factors which may influence the stability of the resulting complex or the suitability of the administered oligomer.

There are at least four major basic strategies for triple helix formation disclosed below. Two of these employ oligomers which contain inverted polarity. Thus, the invention provides oligonucleotides which have inverted polarities for at least two regions of the oligonucleotide. By "inverted polarity" is meant that the oligonucleotide contains tandem sequences which have opposite polarity, i.e., one having polarity 5'→3' followed by another with polarity 3'→5', or vice versa. This implies that these sequences are joined by linkages which can be thought of as effectively a 3'-3' internucleotide junction, (however the linkage is accomplished), or effectively a 5'-5' internucleotide junction. Such oligomers have been suggested as by-products of reactions to obtain cyclic oligonucleotides by Capobianco, M.L., et al., Nucleic Acids Res (1990) 18:2661-2669. Compositions of "parallel-stranded DNA" designed to form hairpins secured with AT linkages using either a 3'-3' inversion or a 5'-5' inversion have been synthesized by van de Sande, J.H., et al., Science (1988) 241:551-557. In addition, triple helix formation using an oligomer which contains an effective 3'-3' linkage has been described by Horne, D.A. and Dervan, P.B., J Am Chem Soc (1990) 112:2435-2437.

using a constant motif or by maintaining the polarity constant and changing the binding motif.

Thus, in some instances, the ability of oligonucleotide sequences to hybridize to double-stranded duplex DNA is enhanced by providing oligonucleotides with inverted polarity either so that the binding oligonucleotide can skip from one complementary strand in the duplex to the other as its polarity shifts, or so that advantage can be taken of the alternate motif. In its simplest embodiment, there is a single inversion of polarity in the binding oligonucleotide; of course, inversions can be inserted in a number depending on the DNA duplex target sequence.

Thus, in one aspect, the invention is directed to oligonucleotide sequences containing at least two tandem sequences of opposite polarities and thus at least one linkage which inverts the polarity of the oligonucleotide, and to methods of preparing and using these oligonucleotides. The inversion of polarity may, if desired, be combined with an alternation in the binding motifs with regard to triple helix formation.

The invention also comprises methods for binding an oligonucleotide to tandem portions of both strands of a double-helical polynucleotide duplex comprising the step of coupling the target double-helical polynucleotide duplex with an oligonucleotide to form a triplex, wherein the oligonucleotide is characterized by a first sequence of nucleotides capable to bind a portion of the first strand of the duplex, followed by a second sequence of nucleotides capable to bind a portion on the second strand of the duplex which is proximal to said target portion on the first strand wherein the second sequence has either opposite polarity or an alternate binding motif to the first sequence.

The term "nucleoside" or "nucleotide" will similarly be generic to ribonucleosides or ribonucleotides, deoxyribonucleosides or deoxyribonucleotides, or to any other nucleoside which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. Thus, the stereochemistry of the sugar carbons may be other than that of D-ribose in certain limited residues, as further described below.

"Nucleoside" and "nucleotide" include those moieties which contain not only the known purine and pyrimidine bases, but also heterocyclic bases which have been modified. Such modifications include alkylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles. Such "analogous purines" and "analogous pyrimidines" are those generally known in the art, many of which are used as chemotherapeutic agents. An exemplary but not exhaustive list includes 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylamino-methyl-2-thiouracil, 5-carboxymethylaminomethyl uracil, dihydrouracil, inosine, N6-isopentenyl-adenine, 1-methyl-adenine, 1-methylpseudouracil, 1-methylguanine, 1-methyl-inosine, 2,2-dimethylguanine, 2-methyladenine, 2-methyl-guanine, 3-methylcytosine, 5-methylcytosine, N6-methyl-adenine, 7-methylguanine, 5-methylaminomethyl uracil, 5-methoxy aminomethyl-2-thiouracil, beta-D-mannosyl-queosine, 5'methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, wybutoxosine, pseudouracil, queosine, 2-thio cytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine. "Nucleosides" or "nucleotides" also include those which contain modifications in the sugar

to adjacent nucleotides through -O- or -S-. Not all such linkages in the same oligomer need to be identical.

Inversions of polarity can also occur in "derivatives" of oligonucleotides. "Derivatives" of the oligomers include those conventionally recognized in the art. For instance, the oligonucleotides may be covalently linked to various moieties such as intercalators, substances which interact specifically with the minor groove of the DNA double helix and other arbitrarily chosen conjugates, such as labels (radioactive, fluorescent, enzyme, etc.). These additional moieties may be derivatized through any convenient linkage. For example, intercalators, such as acridine can be linked through any available -OH or -SH, e.g., at the terminal 5' position of RNA or DNA, the 2' positions of RNA, or an OH, NH_2, COOH or SH engineered into the 5 position of pyrimidines, e.g., instead of the 5 methyl of cytosine, a derivatized form which contains, for example, $-CH_2CH_2NH_2$, $-CH_2CH_2CH_2OH$ or $-CH_2CH_2CH_2SH$ in the 5 position. A wide variety of substituents can be attached, including those bound through conventional linkages. The indicated -OH moieties in the oligomers may be replaced by phosphonate groups, protected by standard protecting groups, or activated to prepare additional linkages to other nucleotides, or may be bound to the conjugated substituent. The 5' terminal OH may be phosphorylated; the 2'-OH or OH substituents at the 3' terminus may also be phosphorylated. The hydroxyls may also be derivatized to standard protecting groups.

Oligonucleotides or the segments thereof of 5'→3' or 3'→5' polarity are conventionally synthesized. Methods for such synthesis are found, for example, in Froehler, B., et al., Nucleic Acids Research (1986) 14:5399-5467; Nucleic Acids Research (1988) 16:4831-

and generally by methods known in the art. Nonphosphorous based linkages may also be used, such as the formacetal type linkages described and claimed in copending applications U.S. Serial Nos. 426,626 and 448,914, filed on 24 October 1989 and 11 December 1989, both assigned to the same assignee and both incorporated herein by reference.

Thus, to obtain an oligonucleotide segment which has a 3'→5' polarity, a nucleotide protected at the 5' position and containing an activated phosphite/phosphate group at the 3' position is reacted with the hydroxyl at the 5' position of a nucleoside coupled to a solid support through its 3'-hydroxyl. The resulting condensed oligomer is deprotected and the reaction repeated with an additional 5'-protected, 3-'phosphite/phosphate activated nucleotide. Conversely, to obtain an oligomeric segment of 5'→3' polarity, a nucleotide protected in the 3' position and containing an activated phosphite/phosphate in the 5' position is reacted with a nucleotide oligomer or nucleoside attached to a solid support through the 5' position, leaving the 3'-hydroxyl available to react. Similarly, after condensation of the incoming nucleotide, the 3' group is deprotected and reacted with an additional 3'-protected, 5'-activated nucleotide. The sequence is continued until the desired number of nucleotides have been added.

In addition to employing these very convenient and now most commonly used, solid phase synthesis techniques, oligonucleotides may also be synthesized using solution phase methods such as triester synthesis. These methods are workable, but in general, less efficient for oligonucleotides of any substantial length.

The oligonucleotides of the invention which are designed to target duplexes for triplex formation may have, as stated above, either uniform or mixed motifs and

In their most general form, the inverted polarity oligonucleotides of the invention contain at least one segment along their length of the formula:

3'-----→5'--C--5'-----3' (1)

or

5'-----→3'--C--3'-----5' (2)

where -C- symbolizes any method of coupling the nucleotide sequences of opposite polarity.

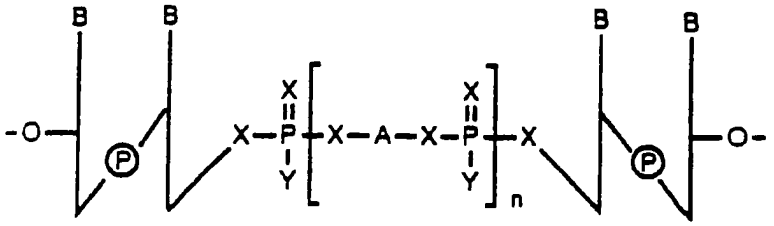
In these formulas, the symbol 3'----5' indicates a stretch of oligomer in which the linkages are consistently formed between the 5' hydroxyl of the ribosyl residue of the nucleotide to the left with the 3' hydroxyl of the ribosyl residue of the nucleotide to the right, thus leaving the 5' hydroxyl of the rightmost nucleotide ribosyl residue free for additional conjugation. Analogously, 5'----3' indicates a stretch of oligomer in the opposite orientation wherein the linkages are formed between the 3' hydroxyl of the ribosyl residue of the left nucleotide and the 5' hydroxyl of the ribosyl residue of the nucleotide on the right, thus leaving the 3' hydroxyl of the rightmost nucleotide ribosyl residue free for additional conjugation.

The linkage, symbolized by -C-, may be formed so as to link the 5' hydroxyls of the adjacent ribosyl residues in formula (1) or the 3' hydroxyls of the adjacent ribosyl residues in formula (2), or the "-C-" linkage may conjugate other portions of the adjacent nucleotides so as to link the inverted polarity strands. "-C-" may represent a linker moiety, or simply a covalent bond.

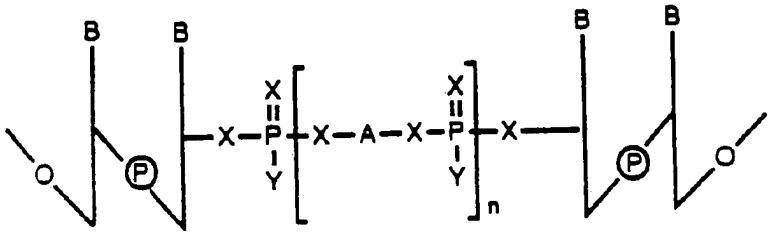
It should be noted that if the linkage between strands of inverted polarity involves a sugar residue,

would be employed if the polarity of the chain were to remain the same. This additional nucleotide may also contain a linker moiety which may be included before or after condensation to extend the chain.

For example, in one illustrative embodiment of the formulas (1) and (2), these compounds include inversion-conferring linkages of the formulas:

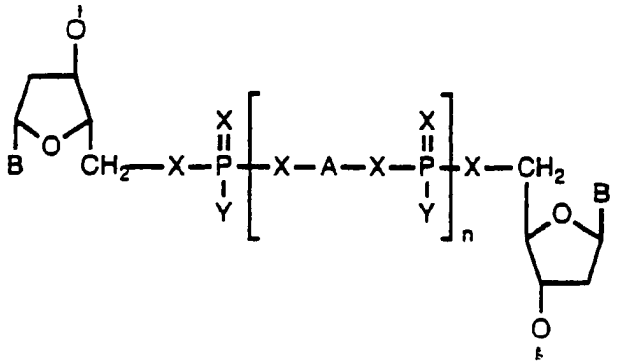


(1')



(2')

or the more complete representation:



(1')

Also, any linkage form can be included using a prelinked dimer in the solid phase sequence.

When n is 0 in the above embodiment, the 3'-3' or 5'-5' linkage is simply formed using standard oligonucleotide synthesis techniques wherein the nucleotide to be added to the sequence is protected and activated in the opposite orientation from that which would be used if the original chain polarity were followed. When n=1, a linker is utilized to effect the inverted polarity linkage. There is no theoretical reason that n cannot be >1; however, generally it is more convenient to limit the synthesis to the intermediation of one linker.

When a linker moiety is employed, the phosphite/phosphate activated linker can be included directly in the continuing oligonucleotide synthesis, followed by coupling to the first nucleotide of the inverted sequence or the first such nucleotide can be supplied already derivatized to the phosphite/ phosphate activated linker. In general, the linker comprises a diol or diamine, the residue of which appears as "A" in formulas 1' and 2'. Thus, in a typical synthesis protocol, one hydroxyl (or amino) of the diol (or diamine) is protected and the other is an activated phosphite/phosphate. This protected form can be coupled to the oligonucleotide chain attached to the solid support and then deprotected and reacted with the subsequent nucleotide residue.

Similar diol or diamine type (or disulfhydryl or hydroxyl/sulfhydryl type) linkers are also convenient when the linkage between inverted polarity segments is to be effected between adjacent bases or between a base and a sugar moiety, or these can be used to link adjacent sugars directly without the inclusion of the phosphodiester or analog thereof. In these instances, it

The linker may also contain unsaturation, so that it may be of the exemplary formulas:

$HOCH_2(CX_2CX_2)_{n3}CH_2OH$, wherein n3 is an integer of 1-7 and each pair of X or adjacent C is independently H or a π bond; or

$HOCH_2(CX_2CX_2)_{n4}CH_2(CX_2CX_2)_{n5}CH_2OH$, wherein n4 and n5 are integers of 0-7 and wherein the sum of n4 and n5 is not greater than 7 and wherein each pair of X or an adjacent C is independently H or together are a > bond.

In these embodiments also, one or more methylene groups may be replaced, provided it is not adjacent to an additional heteroatom, by O, S or NH.

The dihydroxy, diamino or equivalent linker compound may also be cyclic, either non aromatic or aromatic. Nonaromatic embodiments include diols such as cis- or trans-3-4-dihydroxyfuran, cis- or trans-2-hydroxymethyl-3-hydroxyfuran, and cis- or trans-2-hydroxymethyl-4-hydroxyfuran, said furan either further unsubstituted, or further substituted with one or two noninterfering alkyl(1-4C) substituents, or may include N-heterocycles such as piperazine or piperidine.

Linkers containing aromatic rings may include residues of 1,2-dihydroxymethylbenzene; 1,4 dihydroxy methylbenzene; 1,3-dihydroxymethyl benzene; 2,6-dihydroxy methylnaphthalene; 1,5-dihydroxymethylnaphthalene; 1,4-bis(3-hydroxy propenyl)benzene; 1,3-bis(3-hydroxy propenyl)benzene; 1,2-bis(3-hydroxypropenyl) benzene; 2,6-bis(3-hydroxypropenyl)naphthalene; 1,5-bis(3-hydroxypropenyl)naphthalene, 1,4-bis(3-hydroxypropynyl)benzene; 1,3-bis(3-hydroxypropynyl)benzene; 1,2-bis(3-hydroxypropynyl) benzene; 2,6-bis(3-hydroxypropynyl)naphthalene; and 1,5-bis(3-hydroxypropynyl)naphthalene. Figure 1 shows the coupling using 1,4-dihydroxymethylbenzene as it bridges either two ribosyl or two xylosyl residues. As a portion of the inverted

A particular application of a switchback or inverted polarity motif lies in stabilization of oligonucleotides with respect to nuclease degradation. Even a single inversion of nucleotide linkage, preferably at the 3' terminus, will result in enhanced stability. As illustrated in Example 6 below, oligonucleotides with an inverted base moiety at the 3' terminus, either through a phosphodiester or derivatized phosphoramidate linkage exhibit enhanced stability in the presence of fetal calf serum.

Synthesis Methods

For the embodiments of formulas 1' and 2', the synthesis of oligonucleotides having inverted polarity may be accomplished utilizing standard solid phase synthesis methods.

This oligonucleotide chain elongation will proceed in conformance with a predetermined sequence in a series of condensations, each one of which results in the addition of another nucleotide. Prior to the addition of a nucleoside having an activated phosphite/ phosphate, the protecting group on the solid support-bound nucleotide is removed. Typically, for example, removal of the commonly-employed dimethoxytrityl (DMT) group is done by treatment with 2.5% v/v dichloroacetic acid/dichloromethane, although 1% w/v trichloroacetic acid/dichloromethane or $ZnBr_2$-saturated nitromethane, are also useful. Other deprotection procedures suitable for other protecting groups will be apparent to those of ordinary skill in the art. The deprotected nucleoside or oligonucleotide bound to solid support is then reacted with the suitably protected nucleotide containing an activated phosphite/ phosphate. After each cycle the carrier bound nucleotide is preferably washed with anhydrous pyridine/ acetonitrile (1:1, v/v), again

residue and a phosphonate residue in the other. The derivatized linker is coupled to the solid supported strand under standard reagent conditions and then deprotected conventionally. Further standard nucleotide coupling results in extension of the chain in the 3'→5' orientation.

The reactions to illustrate the formation of the 3'-3' coupled synthon used in Figure 2, wherein is exemplified linkage of the 3' positions in adjacent sugar residues through 1,4-dihydroxymethylbenzene (dibromomethylbenzene) is shown in Reaction Scheme 1.

In general, the first step shown is conducted at about 45°C for 24 hours and yields about 80-95% yield of the doubly-protected synthon shown as Formula 1, which is then partially purified through flash chromatography. The second step which constitutes partial deprotection to obtain the monoprotected form of the compound, shown as Formula 2, is conducted at room temperature and takes only about 15 minutes. A mixture of products results, and the crude mixture can be used in the third step of the reaction which yields the phosphonate derivative usable in synthesis. As shown in Figure 2, a 5'→3' nucleotide chain derivatized as solid support is initially reacted with the synthon, followed by deprotection and subsequent reaction with 5' activated 3' protected nucleosides.

If coupling of the inverted portion of the oligonucleotide is through an internucleotide linkage conjugating the bases of adjacent nucleotides or the base of one nucleotide to the ribosyl moiety of the other, or adjacent ribosyl residues through linkages which do not involve activated phosphite/phosphate, it is preferable to form the dimeric nucleotide, which is then included in the synthesis in suitably activated and protected form. For example, adjacent methyl cytosines or thymidines may be linked through the methyl groups at the 5-positions of the pyrimidine rings using a variety of techniques by converting the 5-position to, for example, hydroxymethyl, allyl amine, acrylic acid, or propenyl residues, as is commonly practiced. These reactive groups can then be further coupled through bifunctional linkers or by suitable alternate condensation to obtain dimeric forms of the methyl cytidine or thymidine, or mixed nucleosides. For inclusion of the dimer in the oligonucleotide of inverted polarity, the dimer is protected, if needed, in, for example, both 5' positions and activated in one 3' position and protected in the other for continuation of the synthesis. Extension of the chain continues from the included dimer using nucleosides of inverted protection/activation patterns.

In one example, for a dimer wherein adjacent 5-positions of the bases are linked through $-(CH=CH-CH_2NH)_2CO$, the inclusion of this dimer to obtain a 5'-5' link can be shown diagrammatically in Figure 3 where (S) = polymeric or other solid phase support, Pr_B = a DMT protecting group; Pr_C is a trimethyl acetyl protecting group; P_A = activated phosphite/phosphate; and (P) is as defined above.

Dimers may also be formed between adjacent sugars, and the resulting dimers used as above in standard synthesis. For example, the 3' positions of two

polyethylene glycol, a polypeptide or a lipophilic group is utilized, such a group may facilitate transport of the oligonucleotide through the cell membranes, thus increasing the cellular uptake of the oligonucleotide. A substituent on the amine may also include a group which affects the target DNA to which the oligonucleotide will bind such as providing covalent linkages to the target strand to facilitate cleavage or intercalation of the switchback oligonucleotide to the target strand. The substituents on the amine may contain chromophoric groups such as fluorescein or other labels, including radioactive labels, chelating agents and metal chelated ions, to label the oligonucleotide for identification. The substituents may thus also serve a cutting function (i.e., a site for cutting the duplex) or a receptor function such as a receptor ligand. The substituents on the amine which form the phosphoramidate linkage may thus be virtually any moiety which does not prevent the oligonucleotide from binding to the target duplex.

More than one derivatizing moiety may also be used as two or more phosphoramidate linkages need not contain the same substituents. This may be accomplished by generating a first nucleotide hydrogen phosphonate linkage and then oxidizing it with a first amine, generating a second hydrogen phosphonate linkage and oxidizing it with a second (different) amine.

While the formation of the phosphoramidate linkage provides a convenient method for attaching the groups which derivatize the oligonucleotide to confer useful properties, other methods may also be used. The useful substituents may be attached to the sugar moieties or to the bases, or by any other method generally known in the art.

After completion of the synthesis, the oligonucleotide is separated from the carrier using

oligonucleotides of the invention which maintain the same binding motif but are intended to cross over between the strands of the target duplex, an inversion of polarity will be provided. Thus, the nucleotides will comprise, on one side of the 3'-3' (or 5'-5') inversion, bases which bind to one strand of the duplex according to the motif chosen, with the bases on the other side of the 3'-3' (or 5'-5') junction selected to be bases which will bind to the subsequent bases on the opposite strand of the duplex according to the same motif.

In this manner triple helix recognition may be extended by switching recognition from one strand of the duplex to the other and then back again, if desired. Also, certain nucleases may be blocked, since the oligonucleotides according to the present invention can present ends not recognizable by exonucleases. Thus, oligonucleotides having two 5'-ends, will be resistant to 3'-exonucleases.

Since the switchback oligonucleotides of the invention are intended to expand the strength of binding to duplex DNA, the sequence of nucleotides in each portion of the oligonucleotide is determined by the sequence of bases in the target duplex. For the CT motif, target duplex sequences which contain multiple adenyl residues in a homopurine region of one chain, followed by a region of homopurines comprising guanines in the opposite strand will mandate a switchback oligonucleotide which is polyT in the polarity opposite to the polyA tract followed by polyC in the polarity opposite to that of the polyG tract. Alternating A/G sequences in the first strand of the target duplex will mandate alternating T/C sequences in a first region of oligomer parallel to the enriched purine target, followed by a sequence of inverted polarity which is parallel to the second strand sequential sequence in the duplex.

followed by polyG with 3'→5' orientation, thus switching at the point of inverted polarity, from the CT to the GT motif.

Although, for simplicity, the oligomers of the invention have been described as fitting only one of three "categories"--i.e., inverted polarity maintaining the same motif; inverted polarity correlated with a switch in motif; single polarity and a switch in motif; it is clear that these categories can reside together on a single oligomer whose binding mode to form the triplex thus varies along the targeted duplex. Thus, an inverted polarity segment wherein the inversion is accompanied by a motif switch could be followed by an additional inversion wherein the motif is maintained. In the first inversion, accompanied by the motif switch, a single strand of the duplex will be targeted; at the second inversion wherein the motif is maintained, the oligomer will cross over to recognize the second strand of the duplex.

The sequence design of the oligomers which are intended to cross over between strands of the target duplex must take account of null bases residing in the duplexes which are effectively out of position to participate in the binding. The geometry at present is not completely understood; however, it is clear that the presence of any null bases must be accounted for. The geometry of the double helix results in a spacing requirement so that at a 3'-3' linkage in the oligonucleotide with a CT-CT motif there will be approximately 0-4, probably 1-2 essentially null bases in the duplex; there appear to be no null bases required in the case of the 5'-5' switchback. The opposite is true for combinations of 3'-3' linked GT-GT motif. The null base spacing can be provided by arbitrary nucleotide

invention by sequence-specific orientation in the major groove of the DNA double helix.

In addition to antisense applications, wherein specific sequence recognition is significant, alternate therapeutic mechanisms for oligomers of the invention can be advantageously employed. Such oligomers are generally useful as inhibitors of polymerases such as viral polymerases, to interfere with binding factors to nucleic acids such as transcription initiating or inhibiting factors, to induce the production of interferon endogenously, and so forth. The oligomers of the invention may be administered singly, or combinations of oligomers may be administered for adjacent or distant targets or for combined effects of antisense mechanisms with the foregoing general mechanisms.

In therapeutic applications, the oligomers are utilized in a manner appropriate for antisense therapy in general or to take advantage of the alternate therapeutic mechanisms set forth above. For such therapy, the oligomers can be formulated for a variety of modes of administration, including systemic, topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition. The oligomer active ingredient is generally combined with a carrier such as a diluent or excipient which may include fillers, extenders, binders, wetting agents, disintegrants, surface-active agents, or lubricants, depending on the nature of the mode of administration and dosage forms. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, capsules and suppositories, as well as liquid preparations for injections, including liposome preparations.

may be detected by antibodies which specifically recognize these forms. Means for conducting assays using such oligomers as probes are generally known.

In addition to the foregoing uses, the ability of the oligomers to inhibit gene expression can be verified in in vitro systems by measuring the levels of expression in recombinant systems.

The following examples are provided to illustrate but not to limit the invention.

Example 1

Preparation of 3'-DMT-N^4-benzoyl-dC-5'-H-phosphonate

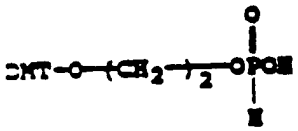
6.4g (10 mmole) of 5'-DMT N^4-benzoyl deoxy-C is dried from 100 ml of pyridine, dissolved into 100 ml of pyridine and to this is added 4g (11.8 mmole) of DMT-Cl and the reaction mixture stirred at room temperature for three days. The reaction mixture is evaporated to approximately half the volume and diluted with 100 ml of CH_2Cl_2, wash with 5% sodium bicarbonate (2 x 100 ml), dry over sodium sulfate and evaporate to dryness. The crude mixture is dissolved into 100 ml of toluene and evaporated to a foam, and this is repeated one more time. The solid is taken up in 50 ml of diethyl ether/50 ml of CH_2Cl_2 and precipitated into 900 ml of hexane at room temperature. The solid is isolated and dissolved into 15 ml of CH_2Cl_2, cool to 0°C and add 100 ml of saturated $ZnBr_2$ in isopropanol/CH_2Cl_2 (15/85) and stirred for 15 minutes. Reaction mixture is quenched into 400 ml of 1M NH_4 OAc, the organic layer separated and wash with $NaHCO_3$ (1 x 200 ml), dry over Na_2SO_4 and evaporate. Purify by silica gel chromatography (CH_2Cl_2/5% MeOH) to yield 50% of the 5'-OH product.

The 5'-OH nucleoside is dried from 50 ml of pyridine then taken up in 10 ml pyridine and 10 ml of

Example 3

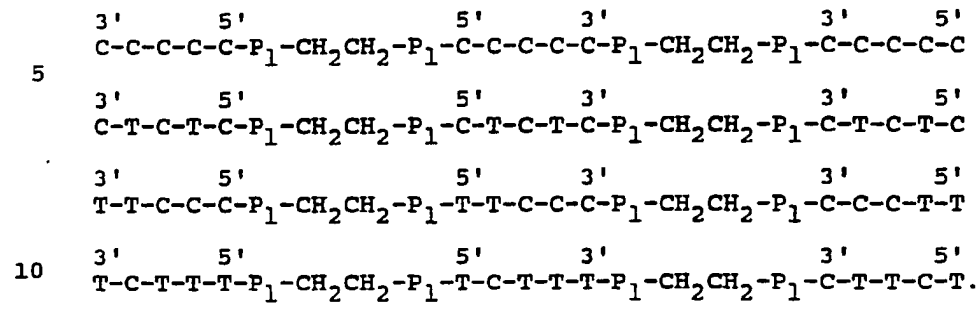
Synthesis of Oligomer Containing Switchbacks

Polynucleotide H-phosphonates condensed at the 3'-end to a solid polymer support are prepared as described by Froehler, et al., Nuc Acids Res (1988) 16:4831-4839; Nuc Acids Res (1986) 14:5399-5467; and Nucleosides and Nucleotides (1987) 6:287-291; using the DBU salt of 5'-protected nucleoside H-phosphonates. After four couplings, one coupling cycle is performed using the ethylene glycol derivative:



The polynucleotide H-phosphonate is then oxidized with aqueous I_2 (0.1M in N-methyl morpholine/water/THF, 5/5/90) to form internucleotide diester linkages. Then five coupling cycles are performed using 3'-protected nucleoside 5'-H-phosphonates, prepared as in Examples 1 and 2. After these couplings the remaining H-phosphonate linkages on the polynucleoside are oxidized with 2-methoxyethylamine in Pyr/CCl_4 (1/5/5), to generate a 10-mer with five diester linkages (one of which is with the ethylene glycol linker) and five phosphoramidate linkages (one of which is with the ethylene glycol linker). The oligomer is removed from the solid support, deprotected with concentrated NH_4OH, purified by HPLC (PRP) using an acetonitrile gradient in 50 mM aqueous TEAP. DMT is removed using 80% HOAc (R.T.) and the solvent is evaporated. The product is desalted, and isolated by evaporation.

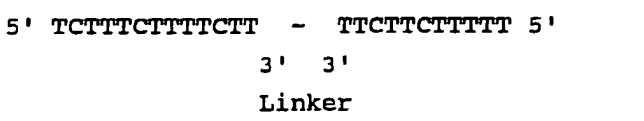
By insertion of an additional linker, the following are prepared:



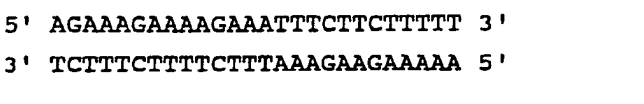
Example 4

Synthesis and Assay of a Recognition Switchback

Using the methods of Example 3, a switchback 24-mer linked with propylene glycol was prepared of the formula:



The duplex DNA to which this oligomer binds has a 26-bp target region having 2 null bases to accommodate the switchback. This target region is of the formula:



To assess the capacity of the switchback oligomer to bind the target region, an assay was conducted as follows. A 144-bp fragment containing four 36-bp cassettes, one of which corresponds to the desired target region, was cloned into pTZ18U, a commercially available cloning vector. The plasmid was cleaved and

5'-TTTTTTNTTTTTNT- ~ -CCCC-5' (1)
3'--3'
linker

which contains a region of inverted polarity but maintains CT motif throughout. This oligomer will effect a crossover between the upper strand wherein the thymine residues target the adenyl-rich portion of the duplex by virtue of the inverted polarity to the polyC tract which targets the polyG region in the opposite strand. (The positions designated "N" in formula (1) can be any nucleotide, as these positions are not responsible for recognition.)

An alternative approach would be to design an oligonucleotide of formula (2) which employs a motif switch from CT to GT to effect the crossover.

5'-TTTTTTNTTTTTNT - GGGG-3' (2)

Figure 4B shows a region of the human cytomegalovirus genome which encodes the 67 kd phosphorylated protein. This region invites similar options.

Figure 4C shows a portion of the gene encoding human IL-1ß. For conditions characterized by an unwanted amount of this lymphokine, triplex formation using oligomer (3) or (4) could be employed.

5'-GGGGGGG - TTTTTTTTTTT-3' (3)

3'-CCCCCCC - TTTTTTTTTT-3' (4)
5'--5'
linker

Oligomer (3) is the converse of oligomer (2); it employs a motif switch to effect a crossing over of the targeted strands. On the other hand, oligomer (4), somewhat

phosphodiester and the methoxyethyl amine (MEA) derivative of a phosphoramidate linkage were obtained. These oligomers were 5' end labeled using gamma 32P ATP and T4 kinase. The labeled oligonucleotide was then ligated to a 10-mer (5'-TCCAGTGATT) using T4 DNA ligase in the presence of the 33-mer template: 5'-TCGCTGATGGAGAAAAAAATCACTGGAGACCTC. The internally labeled 21-mer 5'-TCCAGTGATT32pTTTTTCTCCAT was desalted with the C_8 SPE column.

The internally labeled oligonucleotides were added to serum-containing media in an H938 human T lymphoma cell culture at a concentration of 10 nM either with or without 20 μM of unlabeled oligonucleotide. The degree of degradation was determined at various time points by polyacrylamide gel analysis. Under these conditions, a control 21-mer having no inverted linkage at the 3' terminus had a half-life of approximately 3 hours; however, both the inverted diester and amidate-linked experimental 21-mers had half lives of more than 7 days.

Example 7

Synthesis of Ether-Linked Dinucleoside H-Phosphonates

The meta-xylyl linked analog of the compound of Formula 3 shown in Reaction Scheme 1 hereinabove was synthesized as described in this example. This compound contains a 5' DMT-protected thymidyl residue linked through the 3' position through a 1,3-xylyl residue and to an additional thymidine residue through the 3' position. The second thymidyl is activated at the 5' position with a phosphonate residue.

400 mg (36 mmol) of NaH (60% dispersion in mineral oil) was washed with anhydrous THF (2 x 10 mL), suspended into 20 mL anhydrous THF and to this was added 545 mg (1.0 mmol) of 5'-DMT protected nucleoside with

The 9 x 9 oligonucleotides were designed assuming one, two or three null bases in the duplex. Thus, the oligomer which assumes one null base pair will have the nucleotide sequence

5'-CTTTTTCTT-X-TTTCTTCTT-5'. (7)

The oligomer designed assuming two null base pairs will have the sequence

5'-CTTTTTCTT-X-TTCTTCTTT-5'. (8)

The oligomer assuming three null base pairs will have the sequence

5'-TCTTTTTCT-X-TTCTTCTTT-5'. (9)

In the above sequences, X represents a linker which is either the residue of an ortho, meta or para xylene, the residue of propylene glycol, the residue of 1,2-dideoxyribose or dT. Where the linker is a residue of ortho, meta or para xylene, the thymidyl residues shown on either side of the residue in the formulas may contain either ribose or xylose as the sugar moiety.

Thus, compounds of formulas (7), (8) and (9) were synthesized wherein X is PG, dDR or dT linked through the 3' positions of the adjacent thymidyl residues, or wherein X represents the residue of ortho, meta or para xylene and the thymidyl residues contain either ribose or xylose; in this instance, the "linker" is denoted o-xylose, m-xylose, p-xylose; o-ribose, m-ribose, p-ribose; depending on whether the sugar residues in the adjacent thymidyl residues are ribose or xylose, although the thymidyl residues are shown in the formulas.

These results indicate the nature of the linkage can be designed to accommodate the number of null base pairs in the target.

The binding of the oligomer to the target duplex was also assessed in a manner similar to that set forth in Example 4 as follows. About 0.1 nmol target DNA labeled with about 50,000 cpm of p32 inserted into a host vector was incubated with various concentrations of the test oligonucleotide over a range of 0.1-100 μM in 50 μl reaction buffer (20 mM MES, pH 6.0, 10 mM $MgCl_2$, 100 mM NaCl) for about 1 hour at room temperature to form the triplex. The triplex was treated with 0.2 units DNAseI for 1 min at room temperature and the reaction was stopped with 2 μl 0.5 M ETDA. A 2 μg sample of carrier tRNA was added and the reaction mixture was precipitated with ethanol. The pellet was resuspended in 3 μl 80% formamide, heated for 5 min at 90°C and run on a 6% denaturing polyacrylamide gel to obtain a footprint. Protection by the 9 x 9 mers was shown by absence of cleavage products of the target in the gel.

Example 9

Cooperative Effect of Xylose Linkers

In a manner similar to that set forth in Example 8, above, a 28-mer of the sequence

```
5' AGAAAAAGAAATTTCTTCTTTTTCTTTT 3'
3' TCTTTTTCTTTAAAGAAGAAAAAGAAAA 5'
```

was inserted into the vector for use as a target. The following oligonucleotides were designed to target this duplex assuming two null bases in the duplex.

5'-TTTCTTTTTCTTCTT-"o-xylose"-TCT-5' (10)

Claims

1. An oligonucleotide comprising

a first nucleotide sequence containing at least three nucleotide residues, said sequence having either 3'→5' or 5'→3' polarity, and, coupled thereto,

a second nucleotide sequence containing at least one nucleotide residue, said second sequence having polarity inverted from that of the first sequence.

2. The oligonucleotide of claim 1 which is capable of forming a triplex with a target duplex DNA.

3. The oligonucleotide of claim 1-2 wherein

the 5' position of a nucleotide at the 5' end of the first sequence is coupled to the 5' position of the nucleotide at the 5' end of the second sequence, or wherein

the 3' position of a nucleotide at the 3' end of the first sequence is coupled to the 3' position of the nucleotide at the 3' end of the second sequence, or wherein

the base of the nucleotide at the 5' end of the first sequence is coupled to the base of the nucleotide at the 5' end of the second sequence, or wherein

the base of the nucleotide at the 3' end of the first sequence is coupled to the base of the nucleotide at the 3' end of the second sequence, or wherein

the base of the nucleotide at the 5' end of the first sequence is coupled to the 5' position of the nucleotide at the 5' end of the second sequence, or wherein

the 5' position of the nucleotide at the 5' end of the first sequence is coupled to the base of the

6. The oligonucleotide of claim 4 wherein said linker residue contains at least one cyclic region.

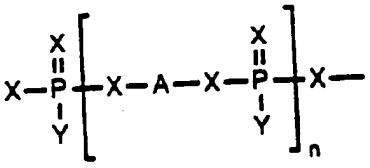
7. The oligonucleotide of claim 6 wherein said cyclic region is nonaromatic.

8. The oligonucleotide of claim 7 wherein said nonaromatic cyclic region comprises a residue of piperidine, piperazine, furan, tetrahydrofuran, cyclohexene, cyclopentene, cyclopentane or cyclohexane.

9. The oligonucleotide of claim 6 wherein said cyclic region is aromatic.

10. The oligonucleotide of claim 9 wherein said aromatic cyclic region is the residue of an o, m, or p disubstituted benzene or disubstituted naphthalene.

11. The oligonucleotide of claim 1-3 wherein said first and second sequence are coupled through a linkage of the formula:



wherein:

Y is H, -OR, -SR, $-NR_2$, O^-, or S^-;

X is O, S, or NR;

19. A method for binding an oligonucleotide to portions of at least one strand of a target double-helical polynucleotide duplex comprising the step of:

contacting a target double-helical polynucleotide duplex with an oligonucleotide under conditions which permit formation of a triplex;

wherein said oligonucleotide comprises a first sequence of nucleotides capable to bind to a first portion of a strand of said duplex, coupled to a second sequence of nucleotides having inverted polarity from said first sequence capable to bind to a proximal second portion of a strand of said duplex.

20. The method of claim 19 wherein said first portion and said second portion of said duplex are on the same strand in said duplex, and wherein one of said first and second sequences in the oligonucleotide is enriched in pyrimidines and the other is enriched in purines.

21. The method of claim 20 wherein the first and second portions of said duplex are on opposite strands of said duplex and said both of said first and second sequences are enriched in pyrimidines or purines.

22. A method for binding an oligonucleotide to portions of both strands of a target double-helical polynucleotide duplex comprising the step of:

contacting said target double-helical polynucleotide duplex with an oligonucleotide under conditions which permit formation of a triplex;

wherein said oligonucleotide comprises a first sequence of nucleotides capable to bind to a first portion of the first strand of said duplex, said first sequence being enriched in pyrimidines, coupled to a second sequence of nucleotides capable to bind to a

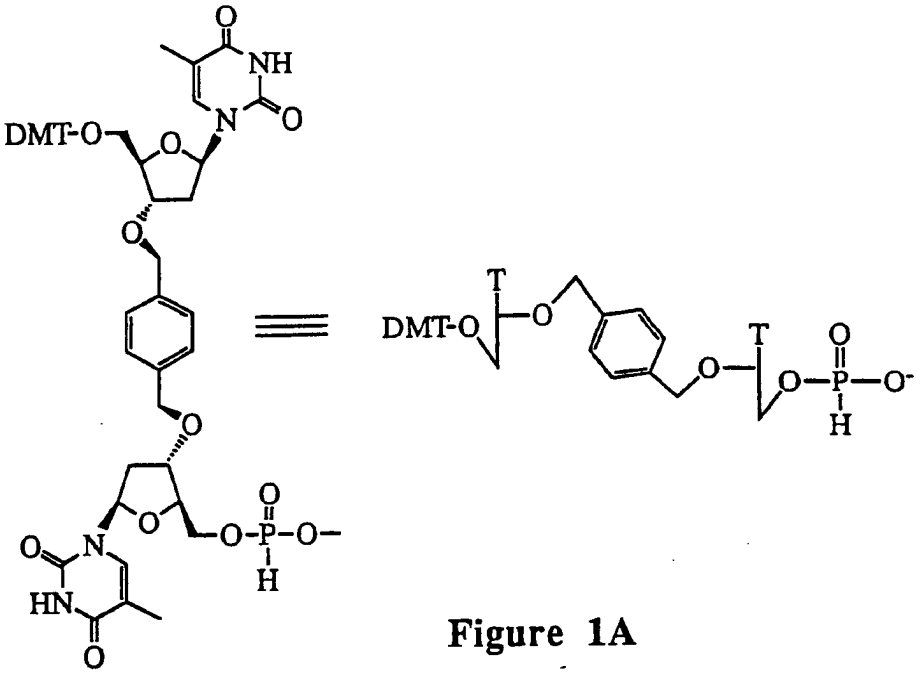


Figure 1A

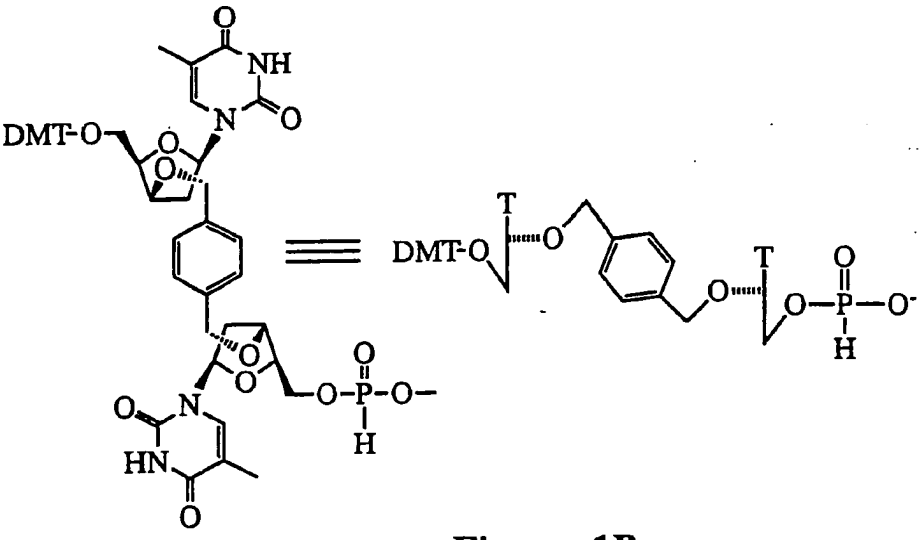


Figure 1B

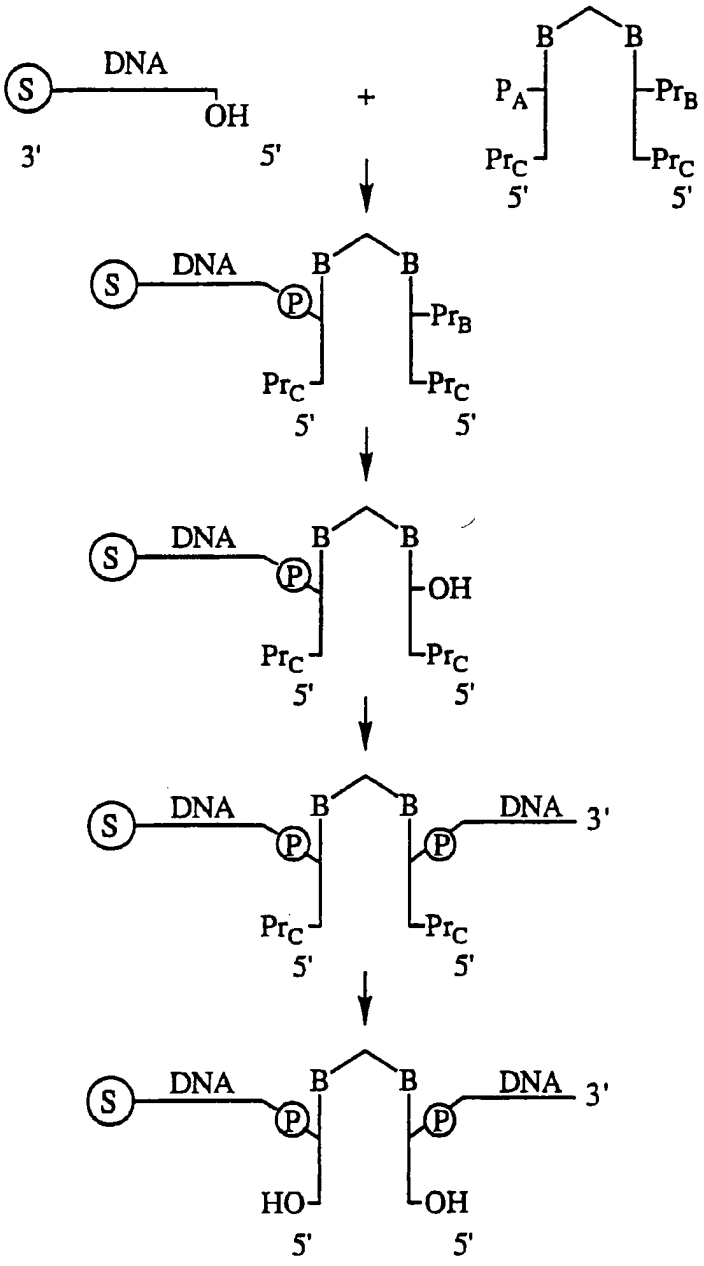


Figure 3

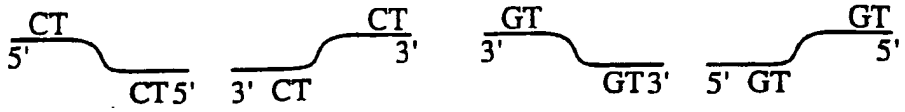


Figure 5A

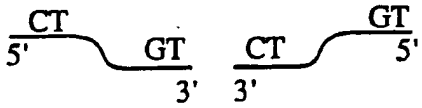


Figure 5B

Figure 5C